# Difference in the Binding Mode of Two Mannose-Binding Proteins: Demonstration of a Selective Minicluster Effect†

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ABSTRACT: Serum-type and liver-type mannose-binding proteins (MBP) are both present in higher animals and both are composed of a carbohydrate-recognition domain (CRD) and a collagenous domain. Although known as mannose-binding proteins, these proteins bind *N*-acetylglucosamine and other related sugars quite well. An earlier specificity study using cloned CRD portions of both types of MBP from rat [Childs, R. A., Feizi, T., Yuen, C.-T., Drickamer, K., & Quesenberry, M. (1990) *J. Biol. Chem. 265*, 20770−20777] revealed that the liver MBP CRD binds the trimannosyl core structure of *N*-glycosides, whereas the serum MBP CRD does not. We studied the substrate preferences of these CRDs using both solid and solution phase assays, testing monosaccharides, glycoproteins, and synthetic cluster ligands. While there was no significant difference in the monosaccharide binding specificities of the two CRDs, they displayed very different affinities for natural glycoproteins and mannose-containing cluster glycosides. Most interestingly, synthetic cluster ligands with two terminal GlcNAc moieties have affinity equal to monovalent GlcNAc ligands toward both CRDs, whereas a series of structurally similar Man-terminated divalent ligand displays about 20-fold enhanced affinity toward liver CRD only. A plausible explanation is that the liver MBP CRD has two sugar binding sites per subunit, one of which binds only mannose, and the other, both mannose and *N*-acetylglucosamine. In contrast, the serum MBP CRD has only one site of the latter type. Results of isothermal titration calorimetry support this hypothesis.

An important class of vertebrate lectins is referred to as C-type, due to calcium-dependency of carbohydrate-binding activity (Drickamer, 1988b). The common motif in this set of lectins is called the carbohydrate-recognition domain, or CRD.[1] Mannose-binding proteins belong to a subclass of the C-type lectins known as the collectins, in which a collagenous domain is attached to the N-terminal end of the CRD (Drickamer & Taylor, 1993). There are two kinds of rat MBP, serum-type and liver-type, which share similar monomer construct but differ in their oligomeric state. The biological role of serum mannose-binding proteins is acknowledged to be as a defense molecule (Summerfield, 1993). Many properties consistent with such a role have been observed in serum MBPs from humans and rodents (Summerfield, 1993). Human serum MBP mRNA levels are found to increase in the liver of trauma patients (Ezekowitz et al., 1988), consistent with the presence of stress response-like control elements in the 5′ region of the gene (Taylor et al., 1989). Immunodeficiencies in children have been linked with absence of serum MBP, and opsonic and complement-fixing properties have been shown in human and rat MBPs (Summerfield, 1993). In contrast, we know little about the function of the liver-type MBP.

Childs et al. (1990) used genetically engineered CRD portions of two rat MBPs, the serum-type (MBP-A) (Drickamer, 1988a) and the liver-type (MBP-C), to examine the fine specificity difference between the two types. The results showed that MBP-C binds the $Man_3GlcNAc_2$ core structure of *N*-glycosides whether the terminal mannoses are occupied or not, whereas MBP-A does not. Since the CRDs possess the same overall structural fold, as was shown by the similarity in three-dimensional structure of the MBP-A and -C CRDs (Ng et al., 1996; Weis et al., 1991b), the mechanism by which ligands are distinguished is of interest. MBP-A CRD has been the subject of extensive structure−function analysis such as interface mapping (Lee et al., 1991), random cassette mutagenesis (Quesenberry & Drickamer, 1992), X-ray crystallography of both ligand-bound and unliganded forms (Weis et al., 1992; Weis et al., 1991b), and protein engineering to alter ligand preference (Drickamer, 1992). By comparison, the binding mechanism of MBP-C, the homologous rat MBP found predominantly in the liver, is less well-studied.

In the present study we determined the binding affinity of a number of structurally well-defined synthetic ligands having two or three terminal monosaccharide residues (Lee & Lee, 1987, 1993). We found, using either direct binding or inhibition assays, that MBP-C CRD consistently bound a series of Man-containing divalent ligands with a 10−20-fold higher affinity than monovalent mannose derivatives, while MBP-A CRD did not show such affinity enhancements, as reported earlier (Lee et al., 1992). These and other binding affinity data are best explained in terms of MBP-C

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[1] Abbreviations: CRD, carbohydrate-recognition domain; MBP, mannose-binding protein; Me O-Man, methyl α-D-mannopyranoside; Me O-GlcNAc, methyl 2-acetamido-2-deoxy-α-D-glucopyranoside; Lyx, lyxose; BSA, bovine serum albumin; PDGF, platelet-derived growth factor; $Le^x$, Lewis X oligosaccharide; ah, 6-aminohexyl; ae, 2-aminoethyl.

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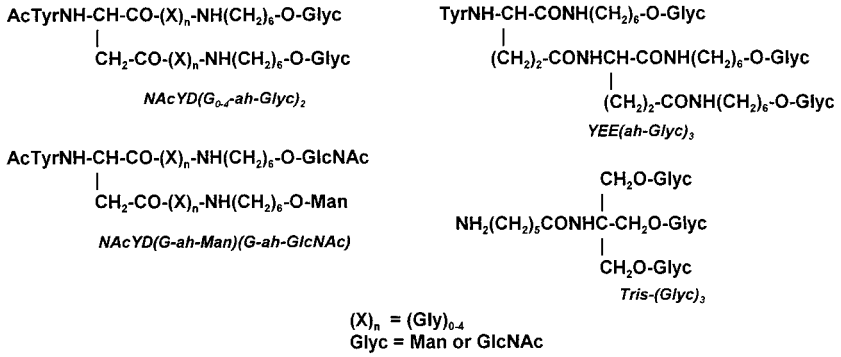
Chart 1




CRD having two Man-binding sites, in contrast to one per subunit for MBP-A CRD.

## MATERIALS AND METHODS

*Materials*. Cluster ligands based on Asp and $\gamma$-glutamylglutamic acid and Tris-based glycosides (Chart 1) were synthesized by the published methods (Lee & Lee, 1987; Lee, 1978). A heterodivalent ligand, NAcYD(G-ah-Man)-(G-ah-GlcNAc) (Chart 1), which has mannose on the $\beta$-carboxyl arm and GlcNAc on the $\alpha$-carboxyl arm, was prepared as described (Lee et al., 1992). A series of mannose-containing bovine serum albumins ($Man_n$-BSA where $n$ is the average number of mannose residues per molecule of BSA) were prepared as described (Stowell & Lee, 1982). Expression and purification of rat MBP-A CRD and MBP-C CRD were performed according to published methods (Drickamer, 1988a), except that cells were lysed in a French press. Immulon 4 Removawell Strips were purchased from Fisher Scientific (Pittsburgh, PA). PDGF in two forms, high-mannose and low-mannose content, was the generous gift of the late Dr. V. Pigiet (Chiron Corp.). Monosaccharides were purchased from Pfanstiehl (Waukegan, IL). All sugars are in the D-configuration (except fucose, which is L), and glycosides are in the pyranoside form. Bicinchoninic acid assay reagents were purchased from Pierce (Rockford, IL). $Na^{125}I$ was from Amersham (Arlington Heights, IL). All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

*General Methods*. Protein concentrations were determined either from absorbance at 280 nm, using an estimated extinction coefficient of 14 308 $M^{-1}$ $cm^{-1}$ for MBP-A CRD and 19 848 $M^{-1}$ $cm^{-1}$ for MBP-C CRD, or by bicinchoninic acid assay (performed according to the manufacturer's recommendation), using BSA as a standard. The concentrations determined by the two methods agreed well.

Radiolabeling of $Man_{30}$-BSA was done by the chloramine T method (Greenwood et al., 1963). Quantification of $^{125}I$ was done using a Packard Minaxi $\gamma$ counter.

Computer modeling of glycoside cluster ligand structures employed the programs Hyperchem and ChemPlus (Autodesk, Inc., Sausalito, CA). The most probable distances between C-4 of the sugar residues were obtained by energy minimization calculations performed according to the manufacturer's recommendation, using the molecular mechanics force field $MM^+$. Conformational searches were done on sequentially selected torsion angles between sugar rings, primarily using default parameters. A water shell was not included in the calculations.

*Microplate Solid-Phase Assay*. The solid phase assay was similar to that described (Quesenberry & Drickamer, 1992). All determinations were done in duplicate, all washes and incubations were done at 4 °C, and the buffer used was a Tris buffer (25 mM Tris at pH 7.8 containing 1.25 M NaCl and 25 mM $CaCl_2$). Briefly, polystyrene wells (Immulon 4 Removawell Strips) were coated with 50 mL of CRD solution per well by overnight incubation, followed by blocking with 1% BSA for 2 h. Blocking solution was then replaced with a solution containing ca. 50 000 cpm of $^{125}I$-$Man_{30}$-BSA (ca. 2000 cpm/ng) and a dilution series of test ligand in the Tris buffer containing 1% BSA. After a 20-h incubation, the liquid in the wells was removed, the wells were then washed, and the $^{125}I$-reference ligand remaining in individual wells was counted. Counts remaining in the wells were averaged for identical wells (usually two). After subtraction of background (radioactivity obtained from BSA-blocked but uncoated wells equally treated with the radiolabeled ligand), counts were converted to the fraction of the maximal binding (i.e., counts obtained in the absence of inhibitor) and plotted against inhibitor concentration in logarithmic scale. Typical inhibition curves of sigmoidal shape were observed (Figure 1). The data were fit to a logistic equation using the program Allfit (De Lean et al., 1978) to determine $I_{50}$ values (the concentration of ligand required for 50% inhibition) of test ligands. When insufficient quantities of test ligand prevented extension of the curve to high inhibitor concentrations, $I_{50}$ values were obtained by manual interpolation and are noted as such in the tables.

*Ammonium Sulfate Precipitation Assay*. This assay for MBP-A CRD activity has been described (Lee et al., 1992). The assay for MBP-C CRD is the same, except that the incubation of the reaction mixture was carried out in the cold for 40 min instead of 20 min at 25 °C. Briefly, a mixture containing MBP-C CRD, $^{125}I$-$Man_{28}$-BSA, and a test compound in 0.3 mL of 0.1 M Tris buffer (pH 7.8) containing 1 M NaCl, 50 mM $CaCl_2$, 0.5% BSA, and 0.1% Triton X-100 was incubated on ice for 40 min, then the bound ligand was precipitated by addition of 0.43 mL of saturated ammonium sulfate solution. After 1 h in the cold, the mixture was filtered through a Whatman 934-AH glass-fiber filter, washed, dried, and analyzed for $^{125}I$. The counts bound as a percentage of counts bound in the absence of inhibitor were plotted against inhibitor concentration on a logarithmic scale. The $I_{50}$ values were obtained manually from each sigmoidal inhibition curve. This assay will be referred to as the solution-phase assay.

*Yeast Suspension Assay*. This assay was carried out for MBP-C CRD as described for MBP-A CRD (Lee et al., 1991). Briefly, [$^{125}I$]MBP CRD, a suspension of washed yeast cells and an inhibitor were rotated end-over-end in a

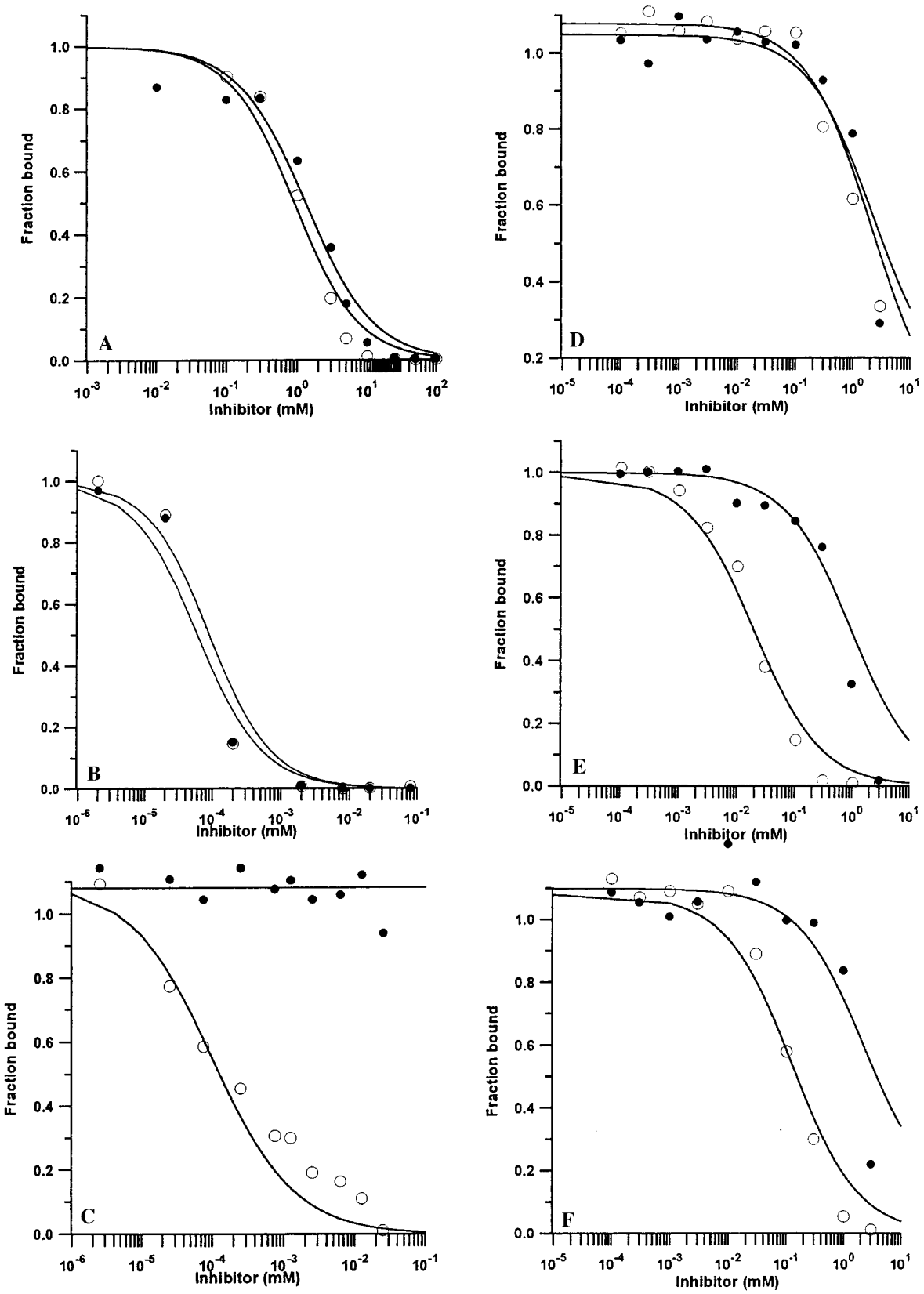



FIGURE 1: Typical inhibition curves obtained by the solid-phase assay. The microplate binding assay was carried out in the presence of various inhibitors at different concentrations. Plots show fitted curves (solid lines) superimposed on the data points (●, MBP-A CRD; ○, MBP-C CRD). Inhibitors used are (*A*) Man, (*B*) invertase, (*C*) porcine thyroglobulin, (*D*) NAcYD(G-ah-GlcNAc)$_2$, (*E*) NAcYD(G-ah-Man)$_2$, and (*F*) NAcYD(G-ah-Man)(G-ah-GlcNAc).

tube at 2 °C for 30 min. [$^{125}$I]MBP CRD bound to yeast cells was determined by centrifugation of the mixture through an oil layer followed by counting the cells collected at the bottom of the centrifuge tube. Data were processed as described for the solution-phase assay. The ligand-binding activity of radioiodinated MBP-C CRD declined measurably within weeks, while MBP-A CRD similarly radioiodinated retained its binding activity almost indefinitely. For this reason, the yeast suspension assay for MBP-C CRD is considered less reliable than the other assay methods.

*Isothermal Titration Calorimetry.* All titration experiments described here were performed in an instrument constructed

at the BioCalorimetry Center (BCC), The Johns Hopkins University (Friere et al., 1990). The instrument is equipped with double 4.92-mL capacity cells, and titrant is delivered to the sample by computer-controlled dual injection syringes driven by a precision stepping motor. Heat generated upon injection is detected by nanovoltmeter-connected thermopiles, and is expressed as power in units of mcal/s. Each titration was accompanied by a series of electrical pulses calibrated to generate heat pulses from 100 to 10 mcal in descending 10-mcal steps. Integration of injection peaks with respect to time using the program ITCArea yield the heat of injection. Summation of injection heats as a function of titrant were normalized to the protein sample concentration using the program. These data were fitted using the program ITCFit to simultaneously determine the stoichiometry, $N$, the association constant, $K_A$, and the enthalpy of the reaction, $\Delta H$. The programs ITCArea, ITCEd, and ITCFit are all the products of the BioCalorimetry Center, The Johns Hopkins University. All experiments described here were carried out at 5 °C in 15 mM glycylglycine buffer, pH 7.8, containing 5 mM $CaCl_2$ and 25 mM NaCl (GCN buffer).

## RESULTS

We have reported the binding site architecture and other binding characteristics of intact MBP-A as well as MBP-A CRD probed by inhibition potencies of many large and small inhibitors (Lee et al., 1991, 1992). In this paper we report the results of similar studies for MBP-C CRD including some new test compounds. In order to carefully compare the binding characteristics of MBP-A and -C CRD, all the compounds were tested side by side using the microplate inhibition assay. Typical inhibition curves obtained by the microplate assay are shown in Figure 1, and the $I_{50}$ values obtained from such plots are summarized in Tables 1 and 2. Since the earlier studies on MBP-A were carried out with a solution assay (ammonium sulfate precipitation assay) and yeast suspension assay, the inhibition potency of a number of compounds for MBP-C was also determined using the latter two assay methods. These results together with earlier data on MBP-A CRD are reported in Table 3.

We tested three categories of inhibitors: monosaccharides and their derivatives; synthetic di- and trivalent glycosides; and macromolecular inhibitors. The results for monosaccharides and their derivatives and macromolecular inhibitors are in Tables 1 and 3, while the results for synthetic di- and trivalent glycosides are shown in Tables 2 and 3. We describe below the results and conclusions obtained with each group of inhibitors under separate subtitles.

*Monosaccharide and Simple Glycosides*. Results in Tables 1 and 3 show that the $I_{50}$ values of small inhibitors obtained by the three assay methods are generally in good agreement, and that the characteristics of monosaccharide binding by MBP-A and -C CRDs are quite similar. As in the case of MBP-A (Lee et al., 1991), MBP-C binds a broad range of monosaccharides (Man, Glc, GlcNAc, and Fuc) with $I_{50}$ values of about 1 mM. Galactose as a free sugar inhibits at about 10-fold higher concentration, but Me O-Gal is not inhibitory at 50 mM. Results with deoxy sugars are also similar to MBP-A in that a lack of hydroxyl group at C-2 (2-deoxy Glc versus Man) or total omission of the C-5 substituent, $CH_2OH$ (Lyx versus Man) lowers the affinity slightly, while both 3-OH and 4-OH are indispensable for binding.

Table 1: $I_{50}$ values for MBP CRDs in the Solid Phase Assay Using Simple Sugars, Glycoproteins, and Neoglycoproteins

| | $I_{50}$ | | relative potency[a] | |
|---|---|---|---|---|
| | MBP-A | MBP-C | MBP-A | MBP-C |
| | (in mM) | | | |
| Gal | 27[b] | 20.3 | 0.07 | 0.05 |
| Lyx | 8.8 | 6.2 | 0.2 | 0.2 |
| Glc | 6.0 | 6.5 | 0.3 | 0.2 |
| GlcNAc | 2.3 | 2.1 | 0.9 | 0.5 |
| Man | 2.0 | 1.1 | 1 | 1 |
| Fuc | 1.2 | 1.8 | 1.7 | 0.6 |
| Me O-Gal | NI (50)[c] | NI (50) | | |
| allyl $\beta$-3-deoxy-GlcNAc | NI (50) | NI (50) | | |
| allyl $\beta$-4-deoxy-GlcNAc | NI (50) | NI (50) | | |
| Man-6-phosphate | 60[b] | 40[b] | 0.03 | 0.03 |
| 2-deoxy-Glc | 3.8 | 6.0 | 0.5 | 0.2 |
| GlcNAc $\beta$1−4GlcNAc | 1.9 | 1.4 | 1 | 0.8 |
| Me O-GlcNAc | 1.1 | 2.1 | 1.8 | 0.5 |
| Me O-Man | 1.0 | 1.5 | 2 | 0.7 |
| Me O-Fuc | 0.6 | 1.2 | 3 | 1 |
| Me S-Man | 0.5 | 0.6 | 4 | 2 |
| | (in $\mu$M) | | | |
| ovalbumin | NI (250) | 100[b] | 0 | 11 |
| soybean agglutinin | NI (250) | 25[b] | 0 | 44 |
| thyroglobulin | NI (25) | 0.13 | 0 | $10^4$ |
| PDGF (low-mannose) | NI (5) | 0.015 | 0 | $10^5$ |
| PDGF (high-mannose) | NI (10) | 0.008[b] | 0 | $10^6$ |
| invertase | 0.6 | 0.1 | $10^4$ | $10^4$ |
| $Man_3$-BSA | 900 | 800 | 2 | 1.4 |
| $Man_{14}$-BSA | 0.09 | 0.1 | $10^5$ | $10^4$ |
| $Man_{23}$-BSA | 0.03 | 0.08 | $10^5$ | $10^5$ |
| $Man_{49}$-BSA | 0.02 | 0.02 | $10^5$ | $10^5$ |

[a] Ratios of Man $I_{50}$ to test compound $I_{50}$ are given as a measure of relative potency. [b] Values are estimated by manual intrapolation of the inhibition curve. [c] Not significantly inhibitory at the concentrations shown in parentheses.

Table 2: $I_{50}$ Values for MBP CRDs in the Solid Phase Assay Using Cluster Glycosides

| | $I_{50}$ (mM) | | maximal inter-sugar distance[a] (Å) |
|---|---|---|---|
| | MBP-A | MBP-C | |
| NAcYD(ae-GlcNAc)$_2$ | 1.6 | 0.8 | 18 |
| NAcYD(ah-GlcNAc)$_2$ | (1)[b] | (0.8) | 26.5 |
| NAcYD(G-ah-GlcNAc)$_2$ | (2.5) | 2.6 | 33 |
| NAcYD(G-G-ah-GlcNAc)$_2$ | 1.2 | 1.2 | 40 |
| NAcYD(G-G-G-ah-GlcNAc)$_2$ | (1.1) | 1.1 | 47 |
| NAcYD(G-ah-Fuc)$_2$ | (0.8) | 0.1 | 33 |
| NAcYD(ah-Man)$_2$ | 4 | 0.095 | 26.5 |
| NAcYD(G-ah-Man)$_2$ | 2 | 0.075 | 33 |
| NAcYD(G-G-ah-Man)$_2$ | 1.8 | 0.065 | 40 |
| NAcYD(G-ah-Man)(G-ah-GlcNAc) | (2) | 0.2 | 33 |
| ZA-tris(Man)$_2$[c] | 2 | 0.46 | 8 |
| MA-tris(Man)$_2$[d] | 5 | 0.34 | 8 |
| YEE(ah-Man)$_3$ | 0.6 | 0.06 | 34 |
| YEE(ah-GlcNAc)$_3$ | 1.1 | 0.65 | 34 |
| Ac-ah-tris(Man)$_3$ | (0.9) | (0.1) | 8 |

[a] From Lee et al. (1989). [b] Values in parentheses are estimated by manual interpolation of the inhibition curve. [c] ZA, *N*-[6-(benzyloxycarbonamido)hexanoyl]. [d] MA, *N*-[5-(methoxycarbonyl)pentanoyl].

*Synthetic Di- and Trivalent Cluster Ligands.* Three groups of synthetic cluster ligands of different design were used. Representative structures of each group are shown in Chart 1. In the first group, a monosaccharide is attached to each carboxylic acid group of Asp *via* an arm of various lengths ranging from aminoethyl (ae) to GlyGlyGly-aminohexyl (GGG-ah). The second group is trivalent glycosides for which $\gamma$-glutamylglutamic acid (EE) is used as the scaffold

Table 3: $I_{50}$ Values for MBP CRDs Obtained by Solution and Yeast Assays

| | solution assay | | | | yeast assay | | | |
|---|---|---|---|---|---|---|---|---|
| | $I_{50}$ | | relative potency[b] | | $I_{50}$ | | relative potency[b] | |
| | MBP-A[a] | MBP-C | MBP-A | MBP-C | MBP-A[a] | MBP-C | MBP-A | MBP-C |
| | (in mM) | | | | (in mM) | | | |
| Man | 1.5 | 1.1 | 1 | 1 | 1.3 | 1.2 | 1 | 1 |
| ManNAc | | | | | 1.1 | | 1.2 | |
| Glc | | | | | 1.8 | 7.4 | 0.7 | 0.2 |
| GlcNAc | 0.9 | 2.3 | 1.7 | 0.5 | 1.5 | 4.5 | 0.9 | 0.3 |
| Gal | | | | | 19.5 | 28 | 0.07 | 0.04 |
| GalNAc | | | | | NI (80)[c] | | 0 | |
| Fuc | 1.0 | 1.9 | 1.5 | 0.6 | 1.1 | 2.2 | 1 | 0.5 |
| 2-deoxy-Glc | | 7.8 | | 0.14 | 2.5 | 8.5 | 0.5 | 0.1 |
| NAcYD(G-ah-GlcNAc)$_2$ | 0.5 | 0.35 | 3 | 3 | 0.5 | 1.15 | 3 | 1 |
| NAcYD(GG-ah-GlcNAc)$_2$ | 0.7 | 0.25 | 2 | 4 | | 0.9 | | 1.2 |
| NAcYD(ah-Man)$_2$ | 1.45 | 0.061 | 18 | 0.9 | 0.13 | 1.4 | 9 | |
| NAcYD(G-ah-Man)$_2$ | 0.56 | 0.05 | 3 | 22 | 0.12 | 0.17 | 11 | 7 |
| NAcYD(GG-ah-Man)$_2$ | 0.65 | 0.07 | 2 | 16 | | 0.13 | | 9 |
| NAcYD(G-ah-Man)(G-ah-GlcNAc) | 0.44 | 0.14 | 3 | 8 | 0.55 | 0.28 | 2 | 4 |
| YEE(ah-Man)$_3$ | 0.75 | 0.035 | 2 | 31 | 0.55 | 0.09 | 2 | 13 |
| | (in $\mu$M) | | | | (in $\mu$M) | | | |
| ovalbumin | 2.7 | 2.7 | 600 | 400 | NI (98) | | 0 | |
| thyroglobulin | 0.01 | 0.018 | $10^5$ | $10^5$ | NI (8.3) | 5 | 0 | 24 |
| invertase | 0.01 | $5.6 \times 10^{-4}$ | $10^5$ | $10^6$ | 0.1 | 0.74 | $10^4$ | $10^3$ |
| Man$_4$-BSA | 0.2 | 0.7 | $10^4$ | $10^3$ | 10 | | $10^2$ | |
| Man$_{15}$-BSA | 0.2 | 0.05 | $10^4$ | $10^4$ | 2 | 30 | $10^3$ | 40 |
| Man$_{20}$-BSA | 0.002 | 0.005 | $10^6$ | $10^5$ | 0.03 | 2 | $10^4$ | $10^3$ |
| Man$_{46}$-BSA | 0.003 | 0.005 | $10^6$ | $10^5$ | 0.02 | 0.8 | $10^5$ | $10^3$ |

[a] $I_{50}$ values for MBP-A CRD are from Lee et al. (1991, 1992). [b] Ratios of Man $I_{50}$ to test compound $I_{50}$ are given as a measure of relative potency. [c] NI, not inhibitory at the concentrations shown in parentheses.

in a similar design. Compounds in the third group are *O*-glycosylation products of *tris*(hydroxymethyl)aminomethane derivatives, and as such have a very short arm of one methylene unit between sugar and the branching point.

These glycosides did not show any significant glycoside cluster effect (affinity enhancement achieved by multivalent ligands over monovalent ones that is greater than would be expected from a simple effect of concentration increase) with MBP-A CRD when tested with the microplate assay (Table 2), confirming the earlier results (Table 3). Similarly, GlcNAc cluster glycosides of this type hardly showed any affinity enhancement for MBP-C CRD. However, all the Man-containing flexible cluster glycosides (i.e., those based on Asp and $\gamma$-GluGlu) showed significant (about 20-fold) affinity enhancement over mannose and monovalent mannosides for MBP-C. A much tighter cluster of two mannose residues as in ZA-tris(Man)$_2$ produced relatively insignificant enhancement factors of about 2−3-fold for MBP-C, but the trivalent cluster of a similar design [Ac-ah-tris(Man)$_3$] may have a small MBP-C-selective cluster effect. The same selectivity of cluster effect was also evident with the solution assay (Table 3). When MBP-C was assayed by the yeast assay, cluster ligands, whether small or macromolecular, generally exhibited smaller affinty enhancement than observed by other methods. This may be related to the instability of the radioiodinated MBP-C. Of all the assays employed, the yeast assay using NAcYD(G-ah-Man)$_2$ as an inhibitor is the only case manifesting a significant cluster effect for MBP-A (Lee et al., 1992). A Fuc-containing divalent glycoside and a hetero-divalent ligand containing one Man and one GlcNAc also showed enhanced affinity (about 10-fold) toward MBP-C but not toward MBP-A.

*Natural and Neoglycoproteins As Inhibitors*. Results of inhibition studies using natural glycoproteins and neoglycoproteins showed less quantitative agreement between the different assays. Comparison of Tables 1 and 3 (excluding the MBP-C results using the yeast assay) shows that the solid-phase assay and the yeast assay generally produced similar $I_{50}$ values, which were much higher than the corresponding values obtained by the solution assay. Within each assay mannose-containing BSAs showed similar affinity for MBP-A and MBP-C CRD, although the actual $I_{50}$ values differ somewhat depending on the assay method. In contrast, except for yeast invertase, most natural glycoproteins tested showed much greater reactivity toward MBP-C than MBP-A. This difference is especially prominent when the solid-phase and yeast assays (Tables 1 and 3) were used, which indicated all glycoproteins with *N*-glycoside structures of non-mannan-type have such low inhibitory potencies toward MBP-A CRD by this assay that the $I_{50}$ values could not even be estimated.

*Isothermal Titration Calorimetry*. The interactions of MBP-A and MBP-C CRD with Me O-Man, Me O-GlcNAc, NAcYD(G-ah-Man)$_2$, and NAcYD(G-ah-GlcNAc)$_2$ were measured by calorimetric titration. The titrations with Me O-Man and Me O-GlcNAc were carried out using a high titrant concentration (80 mM) in order to generate significant amounts of heat. Figure 2 shows a typical titration plot of heat produced versus ligand injection. When the titrations with divalent ligands were carried out using a low concentration (5 mM) of titrant, only the interaction of NAcYD(G-ah-Man)$_2$ with MBP-C CRD gave a measurable heat production. At this low ligand concentration, titration of the same ligand into MBP-A CRD or those of NAcYD(G-ah-GlcNAc)$_2$ into MBP-A and MBP-C CRD all failed to yield a measurable signal. However, meaningful data were obtained when a 10-fold higher concentration (50 mM) of NAcYD(G-ah-Man)$_2$ was titrated into a solution of MBP-A CRD. Due to the much lower solubility of NAcYD(G-ah-GlcNAc)$_2$, titrations at 50 mM could not be carried out for

Table 4: Isothermal Titration Calorimetry Data for MBP-CRDs

| | N | log $K_A$ | $\Delta H^{App}$ (kcal mol$^{-1}$) | $\Delta G^{App}$ (kcal mol$^{-1}$) | $\Delta S^{App}$ (cal mol$^{-1}$ K$^{-1}$) |
|---|---|---|---|---|---|
| MBP-A | | | | | |
| Me O-Man | 1 | 3 | −4.7 | −3.8 | −3.2 |
| Me O-GlcNAc | 1 | 3 | −5.2 | −3.8 | −5.0 |
| NAcYD(G-ah-Man)$_2$ | 1 | 3 | −5.2 | −3.8 | −5.0 |
| MBP-C | | | | | |
| Me O-Man | 1 | 3 | −5.1 | −3.8 | −4.7 |
| | 1 | 0.33 | −0.002 | −0.42 | +1.5 |
| Me O-GlcNAc | 1 | 3 | −4.7 | −3.8 | −3.2 |
| NAcYD(G-ah-Man)$_2$ | 1 | 4.6 | −7.4 | −5.9 | −5.4 |

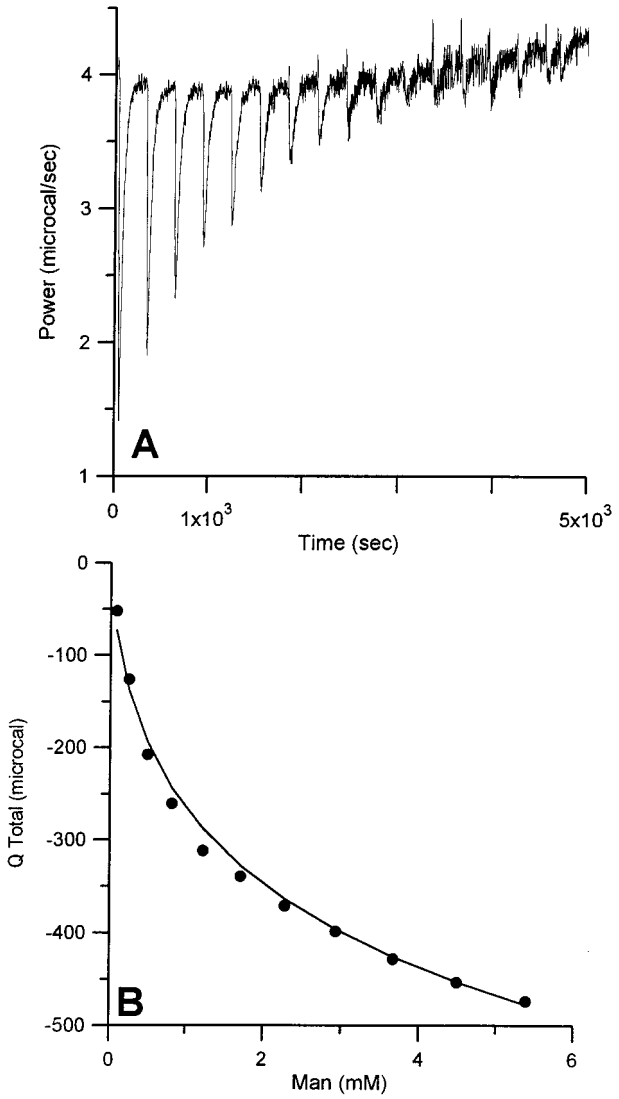



FIGURE 2: Isothermal titration calorimetry measurement of CRD−ligand interactions. (*A*) Power output, in mcal/s, as a function of time is shown for MBP-A CRD + 80 mM Me O-Man, both in GCN buffer. With GCN buffer in the reference cell and MBP-A CRD in the sample cell, 5 $\mu$L of Me O-Man solution was injected every 300 s simultaneously into both cells. (*B*) Cumulative heat, $Q_{Total}$, as a function of ligand concentration accumulated in the cell during the course of the experiment.

this ligand. Thermodynamic parameters obtained from the calorimetric titrations are summarized in Table 4. The $K_D$ values ($1/K_A$) obtained calorimetrically agreed reasonably well with the $I_{50}$ values of the inhibition assay. The enhanced affinity of NAcYD(G-ah-Man)$_2$ for MBP-C CRD over MBP-A CRD translates into ca. 50% increase in the binding energy ($\Delta G^{App} = -5.9$ kcal/mol for MBP-C CRD versus −3.8 kcal/mol for MBP-A CRD). The enhancement is derived mainly from $\Delta H$, since the $\Delta H$ value obtained for the divalent ligand is approximately 1.5 times that measured for the monovalent ligand (Me O-Man).

Fitting of the calorimetric data using the number of binding sites per monomer ($N$) set at 1 or 2 shows that $N = 1$ fits the data as well as $N = 2$ for the binding of Me O-GlcNAc to MBP-A and MBP-C CRD and the binding of Me O-Man to MBP-A CRD. However, the binding of Me O-Man to MBP-C CRD fits better for $N = 2$ than for $N = 1$. The fit with $N = 1$ is excellent for the binding of NAcYD(G-ah-Man)$_2$ to MBP-C CRD, suggesting that the enhanced binding affinity of NAcYD(G-ah-Man)$_2$ to MBP-C CRD involves binding of one molecule of ligand at two sites within the same monomer (see Discussion for details).

## DISCUSSION

MBP-A and -C share homology in their amino acid sequence within the CRD (approximately 55%), as well as in the overall molecular organization of the monomeric unit (Drickamer et al., 1986). However, they do differ in their quaternary structures: serum-type MBPs such as MBP-A exist as large conglomerates of 18−20 monomers with approximate molecular weight of 650 kDa (Oka et al., 1988), whereas liver-type MBPs (e.g., MBP-C) form smaller oligomeric units reaching approximately 200 kDa (six monomers). Although the monosaccharide-binding specificity of the two types of MBP is quite similar in that each binds Man, GlcNAc, and L-Fuc, the two MBPs show different binding preference toward glycoproteins. In fact, the liver-type MBP is also known as the core-specific lectin, because it can bind the trimannosyl core structure of *N*-glycosides (Colley et al., 1988). This difference in the binding specificity is not derived from the difference in their quaternary organization, because the CRD fragments of the two MBPs also show the same specificity difference; i.e., MBP-C CRD binds the trimannosyl core structure of *N*-glycosides, whether its terminal mannosyl residues are occupied or not, whereas MBP-A CRD failed to do so under similar conditions (Childs et al., 1990). These CRDs of MBP-A and -C are approximately 18 kDa in size and include the entire CRD plus some additional polypeptide at the *N*-terminal side but do not contain any repeats of the collagen-like sequence Gly-Xaa-Yaa. Interestingly, however, both fragments appear to exist mainly as trimers (Childs et al., 1990; Weis et al., 1991a). It appears likely, therefore, that the basis for the differential binding of the trimannosyl core structure resides in the CRDs themselves and not in their organization.

A recent report on the X-ray crystallographic structure of MBP-C CRD (Ng et al., 1996) showed that the forces involved in the binding of a mannosyl residue is quite similar

Table 5: Comparison of Binding Affinities of Various Compounds for MBP-CRDs by Different Assays Using Normalized Results[a]

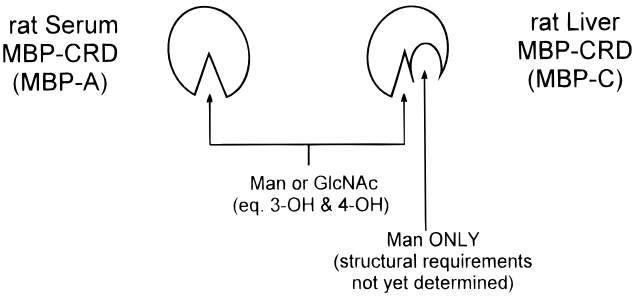
| | relative binding affinity[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | microplate | | solution | | yeast | | calorimetry | | |
| | A | C | A | C | A | C | A | C | ratio[b] |
| Man | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | |
| Glc | 0.3 | 0.2 | | | | 1 | 0.2 | | 0.5 |
| GlcNAc | 1 | 0.5 | 2 | 2 | 1 | 0.3 | 1 | 1 | 0.7 |
| Gal | 0.1 | 0.1 | | | | 0.1 | 0.04 | | 1 |
| Fuc | 2 | 0.6 | 2 | 2 | 1 | 0.5 | | | 0.6 |
| 2-deoxy-Glc | 0.5 | 0.2 | | 0.14 | 0.5 | 0.14 | | | 0.4 |
| ovalbumin | NI[c] | 11 | 600 | 400 | NI | | | | |
| thyroglobulin | NI | $10^2$ | $10^5$ | $10^4$ | NI | 24 | | | |
| invertase | $10^3$ | $10^4$ | $10^5$ | $10^6$ | $10^4$ | $10^3$ | | | 10 |
| $Man_{(3, 4)}$-BSA | 2 | 1 | $10^4$ | $10^3$ | $10^2$ | | | | 0.3 |
| $Man_{(14, 15)}$-BSA | $10^4$ | $10^4$ | $10^4$ | $10^4$ | $10^3$ | 40 | | | 1 |
| $Man_{(20, 23)}$-BSA | $10^3$ | $10^4$ | $10^6$ | $10^5$ | $10^4$ | $10^3$ | | | 5 |
| $Man_{(46, 49)}$-BSA | $10^5$ | $10^5$ | $10^6$ | $10^5$ | $10^5$ | $10^3$ | | | 0.5 |
| $NAcYD(G\text{-}ah\text{-}GlcNAc)_2$ | 1 | 0.4 | 3 | 3 | 3 | 1 | | | 0.7 |
| $NAcYD(GG\text{-}ah\text{-}GlcNAc)_2$ | 2 | 1 | 2 | | | 1 | | | 0.5 |
| $NAcYD(ah\text{-}Man)_2$ | 0.5 | 12 | 1 | 18 | 1 | 9 | | | 21 |
| $NAcYD(G\text{-}ah\text{-}Man)_2$ | 1 | 15 | 3 | 22 | 11 | 7 | 1 | 40 | 11 |
| $NAcYD(GG\text{-}ah\text{-}Man)_2$ | 1 | 17 | 2 | 16 | | 10 | | | 13 |
| NAcYD(G-ah-Man)(G-ah-GlcNAc) | 1 | 6 | 3 | 8 | 2 | 4 | | | 4 |
| $YEE(ah\text{-}Man)_3$ | 3 | 18 | 2 | 31 | 2 | 13 | | | 11 |

[a] The relative binding affinity was determined for all inhibition assays by dividing the $I_{50}$ of Man with that of a given inhibitor. For calorimetry, it is the $K_{A,compound}/K_{A,Me\ O-Man}$. [b] The ratio given is the average of [($I_{50}$ Man/$I_{50}$ test compound) of MBP-C]/[($I_{50}$ Man/$I_{50}$ test compound) of MBP-A]. Due to the limitation in the yeast assay when [$^{125}$I]MBP-C CRD is used, as described in Materials and Methods, data from the yeast assay were omitted in the calculation of this ratio. [c] Found not to be inhibitory at the highest concentrations used.

to that of MBP-A. The most significant and characteristic feature of the binding site of both MBPs is a network of hydrogen bond and coordination bond that connect the 3-OH and 4-OH of mannose with amino acid residues and a calcium ion in the binding site, apparently generating a significant portion of the total binding force. Here we found also that the inhibition profile of the two MBPs are quite similar. Thus, the 3-OH and 4-OH of mannose are absolutely indispensable for the binding activity of MBP-A and -C, and the C-2 and C-5 substituents alter the binding affinity only slightly.

However, a striking difference between these two MBPs is observed when synthetic di- and trivalent ligands were used as inhibitors (Tables 2 and 3). For ease of comparison, binding affinity of compounds relative to mannose obtained by all the inhibition assays and calorimetry was calculated and presented in Table V. From such relative affinity values, affinity enhancement ratios for MBP-C over MBP-A CRD were calculated and presented in the last column of Table 5. This ratio, defined as [($I_{50}$ Man/$I_{50}$ test compound) of MBP-C]/[($I_{50}$ Man/$I_{50}$ test compound) of MBP-A] is close to unity for most small compounds but increases dramatically for the series of flexible Man-containing di- and trivalent ligands. The two important observations follow: (1) MBP-C exhibited 12−20-fold enhancement in affinity with three different Man-containing divalent ligands (differing in the arm length from ah to GG-ah), but not with structurally similar GlcNAc-containing ligands; (2) no such affinity enhancement was observed for MBP-A CRD with a single exception of $NAcYD(G\text{-}ah\text{-}Man)_2$ as inhibitor when assayed by the yeast suspension method (10-fold increase). This phenomenon of selective affinity enhancement for the flexible di- and trivalent Man-containing glycosides was also borne out by the direct binding study using microcalorimetry. Only the interaction of MBP-C CRD with $NAcYD(G\text{-}ah\text{-}Man)_2$ produced the affinity and $-\Delta H$ considerably larger than all other interactions (Table 4).

Scheme 1




Since the cloned MBP fragments each should possess a unique and uniform primary structure, the most plausible explanation for the enhanced affinity of divalent Man-ligands is the presence of two sugar-binding sites on MBP-C monomer: both sites can bind Man, but only one of the sites accommodates GlcNAc (Scheme 1). Simultaneous occupation of the two sites by Man-containing flexible ligands enhances the binding affinity by 10-fold or more. An enhanced binding affinity of a hybrid synthetic cluster ligand consisting of one GlcNAc-terminated arm and one Man-terminated arm for MBP-C CRD (Figure 1F) also agrees with this hypothesis. Since all of the divalent, Man-containing ligands with maximal inter-sugar distance of 25 Å or longer had similar affinity enhancement, the single most important factor in attaining affinity enhancement is probably for a ligand to have long enough arm to allow occupation of two sugar-binding sites simultaneously, rather than its most probable conformation being compatible with the binding geometry. However, since $NAcYD(G\text{-}ah\text{-}Man)_2$ and $NAcYD(G\text{-}ah\text{-}GlcNAc)_2$ have different anomeric configuration, we did determine their most probable conformations by energy minimization, and found them to be similar with ca. 33 Å inter-sugar distance.

We further tried to obtain the binding stoichiometry of MBP-A and -C CRD using direct binding methods. Due to low binding affinity of monovalent ligands ($K_D$ ca. mM),

the Scatchard plots of the data obtained by equilibrium dialysis and the gel filtration method of Hummel and Dreyer (1962) produced a large scatter of data points (not shown). However, these plots strongly suggest that there are two Man-binding sites in MBP-C, one site for Man in MBP-A, and only one site for GlcNAc in both MBP-A and -C. Moreover, fitting of isothermal calorimetric titration data suggest the presence of two Man-binding sites per monomer of MBP-C and one site for MBP-A.

A recent report on the X-ray crystallographic structure of MBP-C CRD (Ng et al., 1996), which appeared after our work was completed, also showed the existence of a secondary binding site for methyl α-mannoside which was only observable in the presence of a very high concentration of the ligand. At the second site only a single amino acid side chain (Lys 130) makes hydrogen bonding with 6-OH, 2-OH, and ring-O of the sugar ring. The involvement of the axial 2-OH of mannoside in the binding process suggests perhaps GlcNAc, lacking this axial OH group, may not bind at the second site. This site is ca. 25 Å away from the first site, and since all three divalent Man-containing ligands with enhanced affinity for MBP-C has two Man residues separated by 25 Å or longer (Table 2), one molecule of the divalent ligands should be able to straddle across both sites simultaneously.

The binding of two terminal Man residues simultaneously at the two Man-binding sites in a single CRD of MBP-C would explain the preferential binding by MBP-C CRD of certain high-mannose type oligosaccharide structures. However, if the second site in our study is the same one observed in the X-ray crystallographic study, the two terminal Man of the trimannosyl core structure should not be able to occupy the two sites simultaneously. Molecular dynamic simulations (Balaji et al., 1994) suggest the Man to Man distances in the trimannosyl core of *N*-glycosides are in the range of 7−9 Å (Drs. V. S. R. Rao and P. K. Qasba, personal communication), which is considerably shorter than 25 Å observed by the X-ray crystallography. Recently we obtained some data which suggest that, unlike MBP-A which interacts with only a single mannosyl residue, the binding site of MBP-C interacts weakly with the second Man residue of mannodisaccharide structures (Lee & Lee, 1997). Perhaps this extended binding area is the reason for the binding of unsubstituted trimannosyl core structure by MBP-C and not by MBP-A. The existence of secondary binding sites has been observed in related C-type CRDs (Geng et al., 1992; Lee & Lee, 1988; Piskarev et al., 1990). A consensus of data including mutagenesis and molecular modeling based on crystal structure data suggests that two separate selectin CRD surface regions are interacting with fucose and sialic acid of the sialyl $Le^x$ structure (Erbe et al., 1992). It may be that MBP CRDs contain a vestigial secondary Man-binding site which degenerated into a less functional site in MBP-A than in MBP-C.

Although MBP-A CRD barely shows any cluster effect toward small synthetic di- and trivalent ligands, it produces large cluster effects of similar magnitude as MBP-C CRD toward neoglycoproteins, such as BSA derivatives containing multiple residues of Man (Tables 1 and 3), GlcNAc, or Fuc (Lee et al., 1991, 1992). Undoubtedly such affinity enhancement is generated by the clustering of three monomeric units of MBP CRDs. Interestingly, when the inhibitor is a macromolecule, the solution assay invariably produced lower $I_{50}$ values than the solid-phase assay or the yeast suspension assay. The difference is especially large when natural glycoproteins were used as inhibitors, and more pronounced for MBP-A than for MBP-C. For instance, the $I_{50}$ of MBP-A CRD for porcine thyroglobulin, a large glycoprotein (660 kDa) with multiple *N*-linked glycans, is 10 nM with the solution assay, whereas 50% inhibition can not be attained using the solid-phase assay. It appears that the high-mannose type oligosaccharide of natural glycoproteins can not easily dislodge $Man_{30}$-BSA once the latter is bound to MBP-A on a solid surface (microplate), nor can it dislodge MBP-A bound to the yeast surface. Although the mechanism involved in this process is not clear, the phenomenon may be relevant to the biological function of MBPs. For MBP-A, a defense molecule which interacts strongly with microorganisms, Man (or other acceptable sugar) residues presented with wide spacing (e.g., neoglycoproteins and yeast cells) may be much preferable to natural glycoproteins with closely clustered sugar residues. In contrast, interactions with natural *N*-glycans may be a requirement in the biological function of MBP-C.

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